LIPOSOME FORMATION USING SUPERCRITICAL ANTI-SOLVENT PROCESSES: DEVELOPMENT OF A CONTINUOUS DENSE GAS PROCESS

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Abstract
Liposomes are phospholipid vesicles. Dense gas processes offer reliable alternatives to conventional methods in liposome formation. The present study is dedicated to the design of a new supercritical process to form liposomes using supercritical CO$_2$. First of all, liposomes have been formed by a two step method using a well known supercritical process (the Supercritical Anti-Solvent or SAS process). Phospholipids have been micronized by the SAS process and then, micronized particles have been hydrated to form spherical and multilamellar liposomes with diameters between 0.1 and 100 µm in a reproducible way. Using the same conditions (the same solvent), the conventional Bangham method did not provide reproducible assay results and formed liposomes were ellipsoidal. Then, we have focused on the design of a new process called Continuous Anti-Solvent or CAS process. Unlike the current dense gas technologies, the CAS process breaks new ground because it is a single step and continuous process. Liposomes prepared with the CAS method are spherical and multilamellar with diameters between 0.1 and 100µm.

INTRODUCTION
Liposomes are spherical vesicles composed of one or more concentric phospholipids bilayers surrounding an aqueous core. Liposomes are classified either by the method of their preparation, the number of bilayers present in the vesicle, or their size. When liposomes are described according to the number of bilayers, they are called unilamellar vesicles (ULV) or multilamellar vesicles (MLV); when liposomes are described according to their size, they are called large unilamellar vesicles (LUV) or small unilamellar vesicles (SUV). Liposome mean diameter range is about 0.02 and 0.1 µm for SUV; about 0.1 and 1 µm for LUV; and, about 0.2 and 100 µm for MLV. Because they are non-toxic and biodegradable, liposomes serve as convenient delivery vehicles for biologically active compounds. However, handling of liposomes in suspension form remains a complex issue given the liposome dynamic behaviour in solution. Many methods have so far been reported for the preparation of liposomes. Conventional methods are the most widely used and especially the Bangham method [1]. Nowadays, the use of these methods is questioned because they suffer from some drawbacks linked to scale-up issues and to the frequent use of toxic organic solvents as ethers (isopropyl ether and diethyl ether), methyl alcohol or chlorinated compounds as chloroform[2]. Given the widespread interest in the use of liposomes in medical, pharmaceutical and cosmetic fields, development of new processes using Generally Recognized as Safe (GRAS) solvents such as supercritical CO$_2$ and in compliance with the constraints imposed by Good Manufacturing Practises (GMP) is required. In these perspectives, dense gas techniques have been developed over the last twenty years as alternative solutions to produce liposomes.
This study focuses on the application of the SAS process to produce liposomes in a continuous way. In the past, several works have been dedicated to high pressure micronization of phospholipids with the SAS process. Recently, Lesoin et al. [3] have hydrated micronized phospholipids powder obtained with the SAS process to form liposomes and they have reported the formation of a bimodal population of liposomes, spreading in the range of 0.1 and 100 µm. But, given that micronized phospholipids react spontaneously upon contact with air, micronized material has to be handled with the greatest precautions before the hydration step. The new process, called the continuous anti-solvent (CAS) process [4], affords a solution to this problem since micronization and hydration are both performed under pressure in the same autoclave.

**FIRST RESULTS – Supercritical Anti-Solvent PROCESS**

The first part of this study is dedicated to liposome formation using the semi-continuous supercritical anti-solvent precipitation process, called SAS. Preparing liposomes via SAS process has to be performed in two steps. Firstly, divided phospholipid microparticles are produced from raw soy lecithin through the SAS process. Secondly, microparticles are hydrated at ambient conditions by an aqueous solution (eventually containing a marker to be encapsulated) under stirring. As a reference control for our experiments, the conventional Bangham method [1], is also used to prepare liposomes. According to the results of the study carried out by Lesoin et al. [3], the use of the SAS process appears to be an efficient and environmentally-friendly process to produce liposomes. Efficient because liposome size distribution is included in the range of 0.1 µm to 100 µm (>80 cumulative volume percent) and encapsulation efficiency is about 20%, which corresponds to commonly expected values (to be used in drug carrier, a liposome size of 2 – 5 µm yields optimal benefit); and environmentally-friendly because this technique enables to use “soft” organic solvents such as ethyl alcohol compared with the organic solvents used with conventional methods (isopropyl ether, diethyl ether, chloroform and methyl alcohol). Moreover, the SAS process is carried out under mild temperature conditions unlike the Bangham method (308 K for the SAS process and 323 K for the Bangham process) which constitutes an asset when liposomes are employed as drug delivery system.

**MATERIALS AND METHODS**

**Materials**

Soy lecithin S75 (71% phosphatidylcholine) and S100 (94% phosphatidylcholine + 6% others (mainly phospholipids)) were purchased from LIPOID (Ludwigshafen, Germany). Analytical grade analysis ethyl alcohol was obtained from Carlo Erba (99.8%). Instrument grade carbon dioxide (purity of 99.7%) from Air Liquide Méditerranée (Vitrolles, France) was used. Distilled water was produced in our laboratory.

**Experimental set-up for the CAS process**

Figure 1 shows a scheme of the experimental set-up for the CAS process. It is composed of a stainless steel high pressure autoclave manufactured by New Ways of Analytics (Germany). It can resist to pressures up to 15 MPa and temperatures up to 318 K. Its volume is of 0.763 L and phase visualization is accessible through two borosilicate windows (situated on the
autoclave sides). For some experiments, pressures higher than 15 MPa were required and then, a windowless autoclave (stainless steel high pressure autoclave, 650 cm³, Fedegari) was employed. The autoclave was supplied by CO₂, organic solution and water. CO₂ was cooled with a cooler (1) and pumped with a volumetric pump (2) (Dosapro Milton Roy, Pont-Saint-Pierre, France). CO₂ was heated to the desired operating temperature with a heater (3). The spraying of the liquid organic solution composed of the dissolved solute (soy lecithin) and of the organic solvent (absolute ethyl alcohol) was performed through a capillary tube (127 µm I.D., Chrompack, Les Ulis, France) with a volumetric pump (2) (Gilson 307, Villiers le Bel, France). The water injection was realized with a third volumetric pump (2) (Gilson 307, Villiers le Bel, France). A 6-bladed Rushton turbine (9) ensures the mixing of the liquid phase in the autoclave (diameter 4 mm). Liposomal suspension is continuously drained off by the bottom of the autoclave (10). As concerns the CO₂ exit, CO₂ is extracted by the bottom of the autoclave (10) as the liposomal suspension. Finally, video recordings are taken with a high speed camera Pulnix TM-6CN, with a capture speed up to 25 frames per second with an exposition time of 1/2000 s. The video recording enables visualization of the dispersion in the autoclave.

Figure 1: Experimental set-up of the CAS process

Procedure

CAS is a single exit process: CO₂ and liposomal suspension are extracted from the bottom of the autoclave (10). A given quantity of aqueous phase was injected in the autoclave. Then, the autoclave was filled with CO₂ and the organic solution was sprayed while the liquid phase was stirred. When the liquid phase looks homogeneous and whitish, the valve (10) was opened in order to obtain the desired CO₂ flow rate. Liposomal suspension was recovered
thanks to the same valve (10). Water was injected continuously to compensate the extraction of liposomal suspension. All experiments were carried out at 308 K, with a solute concentration of 15 wt%, with an organic solution flow rate of 240 mL·h⁻¹ and absolute ethanol as solvent. Process control (water flow rate and CO₂/liposomal suspension exit) is done manually by visual monitoring of the emulsion level in the autoclave.

**Characterization**

The samples of liposomal suspension were stored at 277 K. Liposomes were observed with a phase contrast microscope Reichert-Jung Polyvar (plan objective 4x/10x/25x/40x) to characterize their morphology, shape and size. The volume particle size and size distribution of hydrated microparticles or liposomes were measured by laser diffraction using a Master Sizer S system (Malvern, France).

**RESULTS**

**Liposome size**

Liposomes were prepared with the CAS process and size distribution is presented in Figure 2 \((P = 9 \text{ MPa}, \text{ CO}_2 \text{ flow rate} = 300 \text{ g h}^{-1}, \text{ stirring speed} = 225 \text{ rpm}, \text{ initial quantity of water loaded in the autoclave} = 158 \text{ g and water flow rate} = 180 \text{ mL h}^{-1}\)). An unimodal size distribution is observed with an important proportion of large liposomes (between 10 and 100 μm): 88.56% (cumulative volume percent). Reproducibility was checked (Trial 2 and 3 in Figure 2).

![Figure 2: Liposome size distribution curves](image)

**Phase behavior visualizations**

Changes in the composition of the CO₂/water/surfactant/ethanol quaternary system happened according to experimental conditions (phase flow rate, concentration…). Phase visualization is realized through the windows of the autoclave. Characterization of the emulsion formation and destabilization is done from a qualitative point of view. In Figure 3, images of the phase behavior in the vessel during the injection time show an homogeneous and whitish CO₂-in-water emulsion in equilibrium with a CO₂ rich-phase during the whole set up phase (not a
steady state). CAS process enables an effective phase mixing in the autoclave and as a result, liposome size distributions are unimodal.

![Figure 3: Phase behaviour visualization in the autoclave during the set-up phase](image)

**Liposome morphology and pressure influence**

Phase contrast microscope images of liposomes formed with the CAS process ($P = 9$ MPa, CO$_2$ flow rate = 300 g h$^{-1}$, stirring speed = 225 rpm, initial quantity of water loaded in the autoclave = 150 g and water flow rate = 180 mL h$^{-1}$) are presented on Figure 4. Images show large spherical liposomes surrounded by lots of small spherical liposomes. Membrane thickness of the biggest ones is representative of multilamellar liposomes.

Experiments have been performed to study pressure influence on liposome size distribution (respectively at 9, 12 and 20 MPa while organic solution flow rate = 240 mL h$^{-1}$, CO$_2$ flow rate = 350 g h$^{-1}$, stirring speed = 225 rpm, initial quantity of water loaded in the autoclave = 120 g and water flow rate = 150 mL h$^{-1}$). Given the pressure values, the use of a “closed” autoclave (without windows, 650 cm$^3$) was required. Size distribution curves are presented on Figure 4.b. It appears that size distributions are bimodal. Between 0.1 and 1 µm, liposome population remains constant: 22.34% for 9 MPa, 27.49% for 12 MPa and 18.11% for 20 MPa. All things considered, the results make interesting reading but there are no trends that can be reported.

![Figure 4: Image of liposome formed with the CAS process (a) and liposome size distribution as a function of pressure (b)](image)
Removal of the solvent from the liposome suspension

In the CAS process, ethanol of the organic solution is trapped in the aqueous phase which differs from the classical SAS process. Liposome suspension contains ethanol. This section is dedicated to the removal of the ethanol from the liposomal suspension thank to a membrane-assisted process called diafiltration. Diafiltration is a technique that uses ultrafiltration membranes to remove or lower the concentration of salts or solvents (in our case ethanol) from suspensions containing biomolecules such as liposomes. The technique is also called constant volume washing. Permeable membrane filters are used to separate the components of the suspension based on their molecular size. The solution retained by the membrane is known as the retentate. The solution that passes through the membrane is called permeate. Unlike other techniques used for buffer exchange (membrane dialysis and column-based gel filtration), manipulations are reduced with diafiltration. Diafiltration was run with addition of water to replace ethanol and keeps a constant volume of retentate. The cut-off of the membrane (50 kD) was chosen to avoid liposome loss. Permeate was a water-ethanol solution. Stirred diafiltration cell (400 cm$^3$) was used. Permeate weight was assessed continuously. Compressed air flow involved the circulation into the cell. Reduction of ethanol concentration in the retentate is assessed according to chromatography analyses. It appears that after four washing, ethanol was no more detected by the analyser. A particle counter was used to follow the liposome population before and after the diafiltration process. Diafiltration turns out to be an effective method to remove ethanol from the liposomal suspension produced with the CAS process.

CONCLUSION

A new dense gas process has been developed for the production of liposomes: Continuous Anti-Solvent method. Unlike the current dense gas techniques, CAS method is a single step and continuous process. CAS process benefits from the advantage of the SAS process which ensure the fine division of the lipid material. Such comminution of phospholipid particles is a good preliminary step to liposome formation. Finally, liposomes prepared with the CAS process ($P = 9$ MPa, $T = 308$ K, solute concentration = 15 wt.%, organic solution flow rate = 240 mL h$^{-1}$, CO$_2$ flow rate = 300 g h$^{-1}$, stirring speed = 225 rpm, initial quantity of water loaded in the autoclave = 158 g and water flow rate = 180 mL h$^{-1}$) are spherical and multilamellar with diameters up to 100µm (Figure 2).

REFERENCES


